

**Preliminary Amendment**

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Applicant(s): Timothy E. Benson

Serial No.: 09/772,598

Confirmation No.: 2967

Filed: January 30, 2001

For: CRYSTALLIZATION AND STRUCTURE DETERMINATION OF STAPHYLOCOCCUS AUREUS NAD SYNTHETASE

REMARKS

The Examiner is asked to enter the above amendments to the specification. These amendments simply correct typographical errors and add no new matter to the specification.

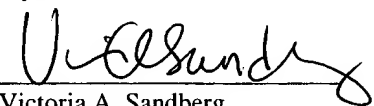
The amendments made on page 34, line 6; and page 43, line 16 correct the titles of the publications cited. The first named authors, volume numbers, page numbers and years of publication were all cited correctly, and from this information the correct titles may be easily found.

The amendment made on page 40, line 23 was made to correct the ending page numbers of the documents cited. The authors, journal titles, volumes, starting page numbers and years of publication were all cited correctly, and from this information the correct page number may be easily found.

The amendment made on page 48, line 31 correct the spelling of the author of the document cited. The journal title, page number and year of publication were all cited correctly, and from this information the correct volume number may be found.

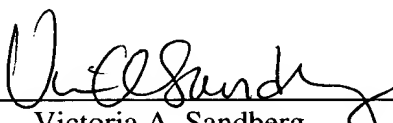
CERTIFICATE UNDER 37 C.F.R. 1.8:

The undersigned hereby certifies that this paper is being deposited in the United States Postal Service, as first class mail, in an envelope addressed to: Assistant Commissioner for Patents, Washington, D.C. 20231, on this 21<sup>st</sup> day of DECEMBER, 2001.

  
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Respectfully submitted for  
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**APPENDIX A - SPECIFICATION/CLAIM AMENDMENTS  
INCLUDING NOTATIONS TO INDICATE CHANGES MADE**

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OF *STAPHYLOCOCCUS AUREUS* NAD SYNTHETASE

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Amendments to the following are indicated by underlining what has been added and bracketing what has been deleted. Additionally, all amendments have been shaded.

**In the Specification**

The paragraph at page 34, lines 4-12, has been amended as follows:

Useful programs to aid one of skill in the art in connecting the individual chemical entities or fragments include, without limitation, CAVEAT (P.A. Bartlett et al., in Molecular Recognition; [in] Chemical and Biological Problems," Special Publ., Royal Chem. Soc., 78:182-196 (1989); G. Lauri et al., J. Comput. Aided Mol. Des. 8:51-66 (1994); available from the University of California, Berkeley, CA); 3D database systems such as ISIS (available from MDL Information Systems, San Leandro, CA; reviewed in Y.C. Martin, J. Med. Chem. 35:2145-2154 (1992)); and HOOK (M.B. Eisen et al., Proteins: Struc., Funct., Genet. 19:199-221 (1994); available from Molecular Simulations, San Diego, CA).

The paragraph at page 34, line 18, has been amended as follows:

*S. aureus* NadE binding compounds may be designed "de novo" using either an empty binding site or optionally including some portion(s) of a known inhibitor(s). There are many de novo ligand design methods including, without limitation, LUDI (H.-J. Bohm, J. Comp. Aid. Molec. Design. 6:61-78 (1992); available from Molecular Simulations Inc., San Diego, CA); LEGEND (Y. Nishibata et al., Tetrahedron, 47:8985-8990 (1991); available from Molecular Simulations Inc., San Diego, CA); LeapFrog (available from Tripos Associates, St. Louis, MO); and SPROUT (V. Gillet et al., J. Comput. Aided Mol. Design 7:127-153 (1993); available from the University of Leeds, UK).

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The paragraph at page 40, line 20 to page 41, line 9, has been amended as follows:

The purified protein was determined to be >96% pure by SDS-PAGE. The sample was received in 25mM Tris, pH 8.0, 5mM  $\beta$ -mercaptoethanol (BME) as requested. BME was added to prevent loss of activity during storage (Zalkin, Methods Enzymol., 113:297-~~301~~~~302~~ (1985)). This buffer system is amenable to crystallization, therefore no buffer exchanges were necessary. The protein was directly concentrated to approx. 20 mg/mL using a pretreated Ultrafree-4 10,000 MWCO concentrator (Millipore). Concentration determination was done by concentration factors based on the original Bradford assay results. Amino acid analysis indicated that the concentration was actually 15 mg/mL, this is a 24% decrease from the earlier calculation. Freshly prepared sample was split into 50  $\mu$ L aliquots, flash frozen in liquid nitrogen, and stored at  $-80^{\circ}\text{C}$ . NAD synthetase was screened using the hanging drop method, in 24 well VDX plates (Hampton Research, Laguna Niguel, CA). The crystallization library consists of Hampton Research Crystal Screen I, Crystal Screen II, and Crystal Screen I- Lite (all available from Hampton Research, Laguna Niguel, CA) and Wizard I, Wizard II, Cryo I, and Cryo II (all available from Emerald Biostructures, Inc., Bainbridge Island, WA). NAD synthetase was screened in all conditions, with hits in Hampton Crystal Screen 1-Lite/43 (15% PEG 1500), Hampton Crystal Screen 1/39 (2% PEG 400, 2.0 M Ammonium Sulfate, 0.1 M Na Hepes pH 7.5), and Wizard 1/41 (30% PEG-3000, CHES pH 9.5).

The paragraph at page 43, lines 14-23, has been amended as follows:

Because these data were of higher quality than the multiple anomalous dispersion data, a molecular replacement solution was initially attempted using the NadE dimer from *Bacillus subtilis* (1nsy.pdb (Rizzi et al., ~~DMBO~~~~EMBO~~ J., 15:5125-34 (1996)). A rotation solution was found using AMORE (Navaza, Acta Cryst., ~~D~~~~A~~50:157-63 (1994)) with a peak height of  $8.6\sigma$  and a subsequent translation solution with a correlation coefficient of 26.9 and an R-factor of 50.3%. Subsequent refinement and rebuilding of this model (and other molecular

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replacement solutions from X-PLOR rotation/translation searches) led to an R-factor of 40% with a Free R-factor above 50%. Since this solution was difficult to refine, further efforts for a structure solution were attempted using the multiple anomalous dispersion data.

The paragraph at page 45, lines 10-17, has been amended as follows:

Molecular replacement experiments were conducted with either AMORE (Navaza, Acta Cryst., [D]A50:157-63 (1994)) or X-PLOR using the *B. subtilis* model of NAD synthetase (PDB id code 1nsy). A portion of the model (residues 106-125) was truncated where the identity of the two proteins was significantly lower than the remainder of the sequence. Using this truncated model, the initial solution for the first dimer gave a correlation coefficient of 18.8 with an R-factor of 51.2%. Searching for the second dimer led to an improved correlation coefficient of 30.0 with an R-factor of 48.5%.

The paragraph at page 48, line 31, has been amended as follows:

Model building was done using the program CHAIN (Sack, J. Molecular Graphics, 6:224-25 (1988)) and LORE (Finzel, Meth. Enzymol., 277:230-42 (1997)). Model for one dimer built using the *B. subtilis* NAD synthetase structure as a reference. The second dimer was placed using non-crystallographic translational symmetry from the molecular replacement solution (R-factor/Free R-factor = 34.1%/39.9%), and refined using positional refinement, torsion angle dynamics and individual B-factor refinement (R-factor/Free R-factor = 24.9%/34.7%). At this stage waters were added and each monomer was thoroughly checked against the electron density. A further rounds of refinement led to the present model (R-factor/Free R-factor = 22.6%/31.2%). All refinement cycles were carried out with XPLOR98 (Brunger, X-PLOR version 3.1, Yale University Press (1992)) incorporating bulk solvent correction during the refinement (Jiang et al., J. Mol. Biol., 243:100-15 (1994)). Progress of the refinement was monitored by a decrease in both the R-factor and Free R-factor. Stereochemistry of the model was checked using PROCHECK (Laskowski et al, J. Appl. Cryst., 26:283-91

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(1993)) revealing no residues in disallowed regions of the Ramachandran plot. Figure 5 was produced in MOLSCRIPT (Kraulis, J. Appl. Cryst., 24:946-50 (1991)) and Raster 3D ([Merritt] Merritt et al., Acta Cryst., D50:869-73 (1994) while Figures 6-8 were produced in MOLSCRIPT (Kraulis, J. Appl. Cryst., 24:946-50 (1991)) alone. Figures 11 and 12 were created in Mosaic-2.